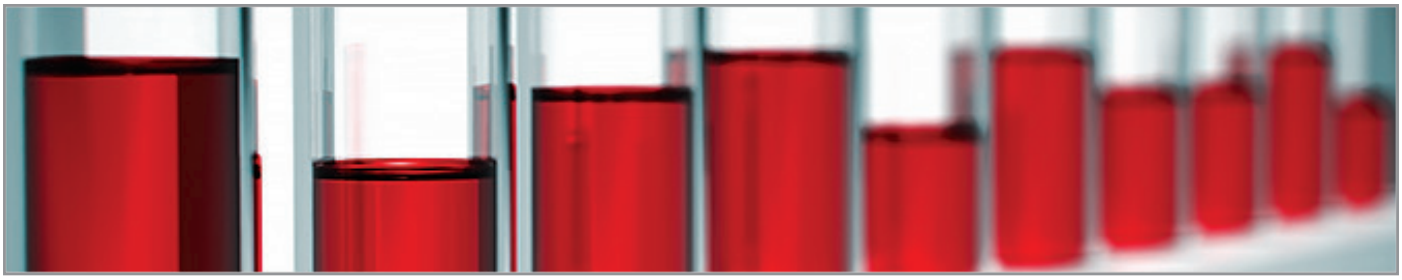


Evaluation of a Simple Method for Methylmalonic Acid Analysis in Human Plasma by LC-MS/MS

By Justin Steimling and Frances Carroll

Abstract

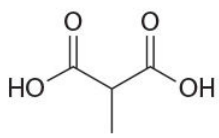
A new LC-MS/MS method for methylmalonic acid (MMA) analysis was developed that provides complete chromatographic resolution from isobaric succinic acid in plasma samples. Excellent chromatographic results were obtained from the direct injection of a protein crash sample supernatant onto a Force C18 column, providing a much simpler sample preparation compared to typical methods.

Introduction

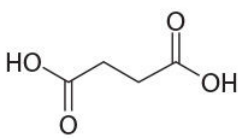
Vitamin B12 deficiency can manifest clinically in a wide variety of physical and behavioral signs and symptoms, but methylmalonic acid (MMA) can be used as a specific biomarker for diagnosis. In the metabolic cycle for energy production, vitamin B12 promotes the conversion of methylmalonyl CoA to succinyl CoA. If there is not enough B12 available, blood levels of MMA begin to rise. High levels of MMA can also result from the metabolic disorder methylmalonic acidemia, which causes the inability to properly digest specific fats and proteins. Therefore, elevated levels of MMA can be used to diagnose functional vitamin B12 deficiency as well as methylmalonic acidemia.

The MMA test typically requires extensive sample pre-treatment incorporating liquid-liquid extraction, derivatization, solvent evaporation, and/or SPE. Additionally, chromatographic resolution can be difficult to achieve between MMA and its naturally occurring isomer, succinic acid. Herein, we present a simple sample preparation method—without derivatization—that allows for the direct injection of protein crash supernatant while still maintaining resolution between MMA and succinic acid in a 5-minute cycle time.

Figure 1: Structures of Methylmalonic Acid and Succinic Acid.



Methylmalonic Acid



Succinic Acid

Experimental

Calibration Standards and Quality Control Samples

Double charcoal stripped human plasma contains endogenous levels of methylmalonic acid; therefore, a surrogate matrix was created by fortifying SeraFlx BIOMATRIX (Cerilliant) at 1000 ng/mL with succinic acid. The surrogate matrix was then spiked with MMA to prepare calibration standards and QC samples. The calibration range was 10–500 ng/mL and surrogate matrix QC samples were prepared at 10, 20, 150, and 400 ng/mL.

The amount of endogenous methylmalonic acid in the double charcoal stripped human plasma was determined to be 13 ng/mL using the calibration standards prepared in the surrogate matrix. Additional QC samples were prepared in double charcoal stripped human plasma by fortifying at the same levels as the surrogate matrix and then quantitatively accounting for the additional endogenous MMA level. The QC samples in human plasma contained combined (endogenous and fortified) MMA levels of 23, 33, 163, and 413 ng/mL.

Internal standard MMA-D3 was prepared at 2,500 ng/mL in water; 5 µL was then added to each sample, and samples were vortexed prior to protein precipitation.

Sample Preparation

Protein precipitation was performed by aliquoting 300 µL of 0.5% formic acid in methanol into 100 µL of sample. The sample was then vortexed for 10 seconds at 3000 rpm followed by centrifugation at 4000 rpm for 10 minutes at 10 °C. The supernatant (250 µL) was then filtered using a Thomson SINGLE StEP standard filter vial (0.2 µm PVDF membrane, Restek cat.# 25895).

Analytical column: Force C18 3 µm, 100 mm x 3.0 mm (cat.# 963431E)
Guard column: Force C18 EXP guard column cartridge (cat.# 963450253)
Mobile phase A: Water, 0.5% formic acid
Mobile phase B: Methanol, 0.5% formic acid
Gradient

Time (min)	%B
0.00	5
0.50	5
3.00	95
3.01	5
5.00	5

Flow rate: 0.7 mL/min
Injection volume: 3 µL
Column temp.: 35 °C
Sample temp.: 10 °C
Ion mode: Negative ESI

Table I: Analyte Transitions for Methylmalonic Acid Analysis.

Compound	Precursor Ion	Product Ion
MMA	117.3	73.1
MMA-D3	120.2	76.2

Results and Discussion

Sample Preparation Evaluation

To ensure that the supernatant was compatible with direct analysis, an initial solvent evaluation was conducted. Trichloroacetic acid (TCA) was initially investigated for use as the precipitating solvent. While using a small volume of 40% TCA (w/v) (25 µL per 100 µL of sample) effectively precipitated proteins, it resulted in no signal for methylmalonic acid, despite dilution with 300 µL of 0.5% formic acid in water prior to analysis (Figure 2). It is speculated that the TCA present in the final extract is not only responsible for the improved peak shape of succinic acid, but also for ion suppression that resulted in reduced sensitivity for MMA. Precipitation with 0.5% formic acid in methanol greatly increased sensitivity compared to TCA when evaluating the same sample (Figure 3). The methanol-based supernatant was compatible with direct analysis providing a sample volume of 3 µL or less was injected in order to avoid solvent effects.

Figure 2: 40% Trichloroacetic Acid (w/v) Protein Precipitation.

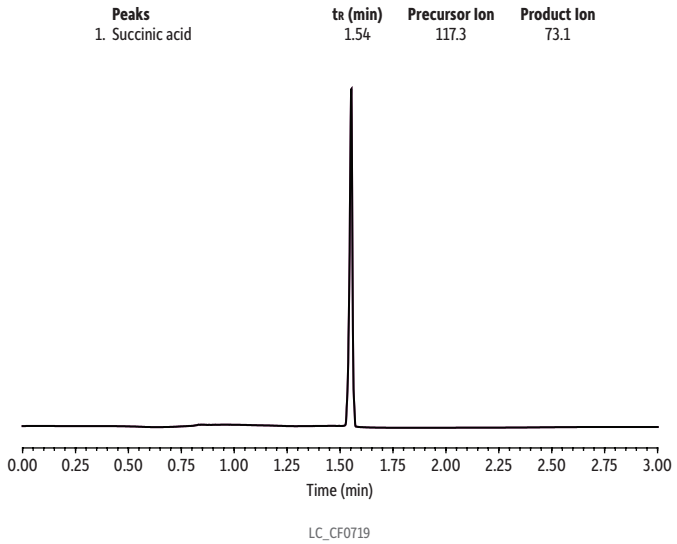
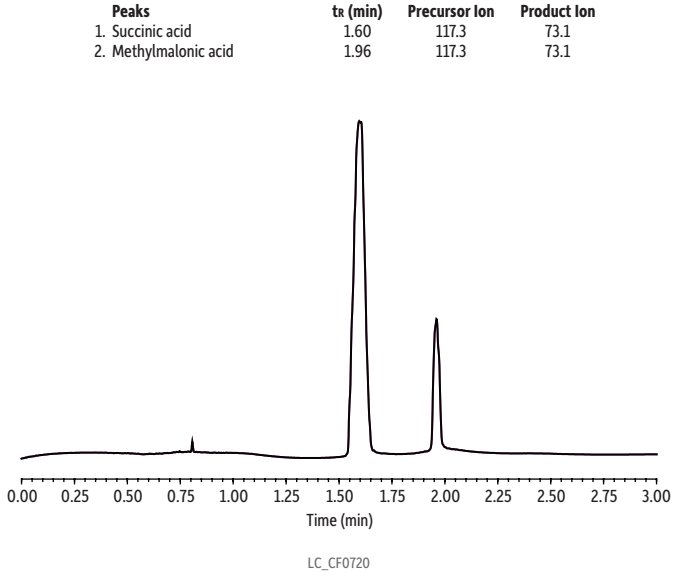


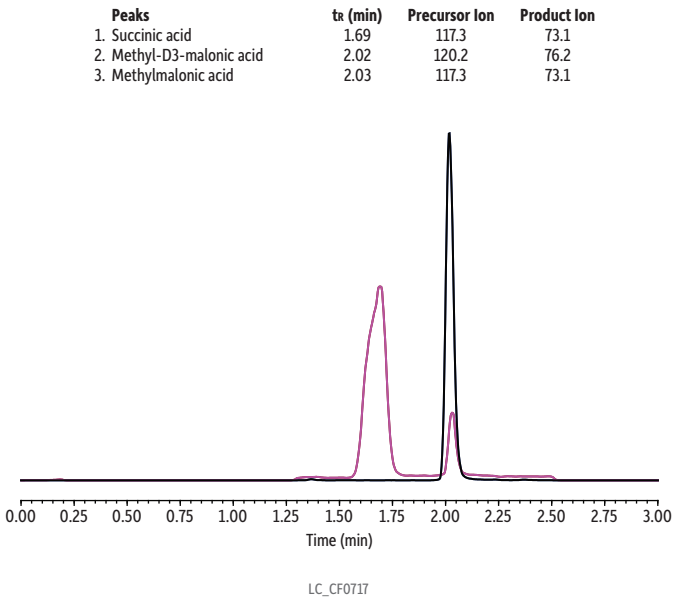
Figure 3: 0.5% Formic Acid in Methanol (v/v) Protein Precipitation.



Chromatographic Performance

A fast, 5-minute analysis of methylmalonic acid (Figure 4) was obtained from the direct injection of the supernatant. MMA was clearly resolved (USP resolution of 3.0) from the isobaric interference, succinic acid, which made peak identification and quantitation easy. While analytical columns containing superficially porous particles can offer a speed advantage for some applications, a fully porous particle Force C18 column was used here because the additional surface area provides the retention required to chromatographically separate MMA from succinic acid.

Figure 4: 50 ng/mL MMA in SeraFlx BIOMATRIX.



Linearity

Using linear 1/x weighted regression, the method showed good linearity for MMA across a range from 10–500 ng/mL with r2 values of at least 0.999 in all three accuracy and precision experiments.

Accuracy & Precision

Precision and accuracy analyses were performed on three different days. The method accuracy was demonstrated to be within 6.00% of the nominal concentration for all QC levels. The %RSD was 5.19%–6.50% and 7.67% for intra- and interday, respectively, at the LLOQ. The %RSD was 0.866%–6.24% and 1.97%–4.99% for intra- and inter-run, respectively, across the low, mid, and high QC levels, indicating good method precision (Table II).

Table II: Interday Accuracy and Precision of SeraFlx BIOMATRIX QC Samples.

Analyte	QC LLOQ (10 ng/mL)			QC Low (20 ng/mL)			QC Mid (150 ng/mL)			QC High (400 ng/mL)		
	Avg. Conc. (ng/mL)	Avg. Accuracy (%)	Precision (%RSD)	Avg. Conc. (ng/mL)	Avg. Accuracy (%)	Precision (%RSD)	Avg. Conc. (ng/mL)	Avg. Accuracy (%)	Precision (%RSD)	Avg. Conc. (ng/mL)	Avg. Accuracy (%)	Precision (%RSD)
MMA	9.40	9.40	7.67	19.0	95.1	4.99	152	101	4.84	404	101	1.97

Matrix Surrogate Evaluation

Because endogenous methylmalonic acid was found in double charcoal stripped human plasma (Figure 5), an evaluation of the use of a surrogate matrix for preparing calibration curves and QC samples was performed. SeraFlx BIOMATRIX has physical and chemical characteristics similar to human serum/plasma and tested negative for the presence of MMA. A calibration curve prepared in SeraFlx BIOMATRIX was used to quantitate the endogenous level of MMA in the double charcoal stripped human plasma. A concentration of 13 ng/mL was determined. The plasma was then spiked with additional MMA at concentrations corresponding to the LLOQ and low, mid, and high QC levels. The method accuracy was demonstrated to be within 7.00% of the nominal concentration for all QC levels. The method precision was 6.77%, which is within the acceptance criteria, for all QC levels, indicating that SeraFlx BIOMATRIX was an appropriate matrix surrogate for the analysis of MMA in human plasma (Table III). The suitability of this surrogate matrix for human plasma can improve lab efficiency by eliminating the need to prescreen control material for detectable levels of MMA.

Figure 5: Endogenous MMA (13 ng/mL) in Double Charcoal Stripped Plasma.

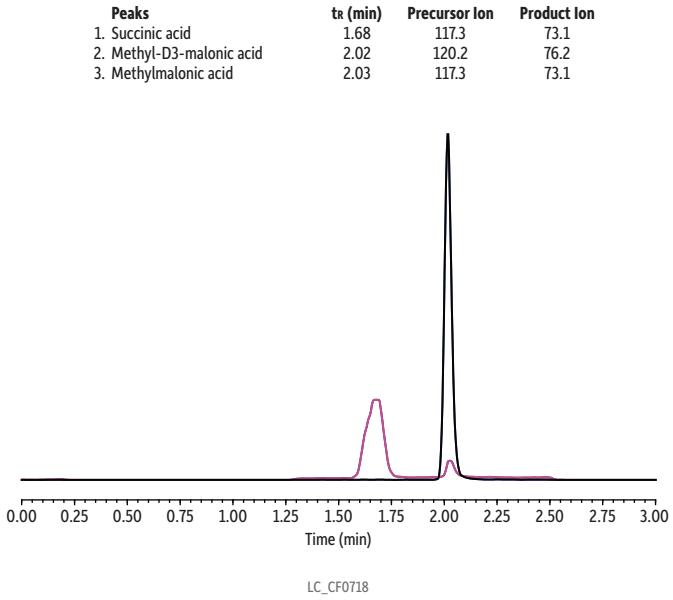


Table III: Inter-Day Accuracy and Precision of Human Plasma QC Samples.

Analyte	QC LLOQ (23 ng/mL)			QC Low (33 ng/mL)			QC Mid (163 ng/mL)			QC High (413 ng/mL)		
	Avg. Conc. (ng/mL)	Avg. Accuracy (%)	Precision (%RSD)	Avg. Conc. (ng/mL)	Avg. Accuracy (%)	Precision (%RSD)	Avg. Conc. (ng/mL)	Avg. Accuracy (%)	Precision (%RSD)	Avg. Conc. (ng/mL)	Avg. Accuracy (%)	Precision (%RSD)
MMA	24.0	104	4.16	34.2	104	6.77	175	107	1.53	421	102	1.34

Conclusion

It was demonstrated that a simple, cost-effective sample preparation method can be used as an alternative to the tedious methods traditionally used for the analysis of MMA. The direct analysis of supernatant reduces costs associated with training, consumables, and extraction time. A Force C18 analytical column is able to successfully separate the naturally occurring isomer of MMA, succinic acid, with good resolution and a fast cycle time of only 5 minutes. SeraFlx BIOMATRIX was found to be a suitable matrix surrogate for the analysis of human plasma, eliminating the need to prescreen control material. With a fast, simple sample preparation procedure and 5 minutes of chromatographic analysis time, the established method provides a high-throughput assay for the clinical diagnosis of vitamin B12 deficiency and methylmalonic acidemia.

Thomson SINGLE StEP Standard Filter Vials with Screw-Top Caps

Catalog No.	Product Name	Cap Color	Material	Porosity	Units
27896	Thomson SINGLE StEP Standard Filter Vials with Screw-Top Caps, 0.2 µm, Nylon w/Preslit Cap, Black Cap, 100-pk.	Black	Nylon	0.2 µm	100-pk.
27897	Thomson SINGLE StEP Standard Filter Vials with Screw-Top Caps, 0.45 µm, Nylon w/Preslit Cap, Pink Cap, 100-pk.	Pink	Nylon	0.45 µm	100-pk.
27895	Thomson SINGLE StEP Standard Filter Vials with Screw-Top Caps, 0.2 µm, PES w/Preslit Cap, Gray Cap, 100-pk.	Gray	PES (polyethersulfone)	0.2 µm	100-pk.
28307	Thomson SINGLE StEP Standard Filter Vials with Screw-Top Caps, 0.2 µm, PTFE w/preslit cap, Green Cap, 100-pk.	Green	PTFE (polytetrafluoroethylene)	0.2 µm	100-pk.
28306	Thomson SINGLE StEP Standard Filter Vials with Screw-Top Caps, 0.45 µm, PTFE w/preslit cap, Blue Cap, 100-pk.	Blue	PTFE (polytetrafluoroethylene)	0.45 µm	100-pk.
27894	Thomson SINGLE StEP Standard Filter Vials with Screw-Top Caps, 0.2 µm, PVDF w/Preslit Cap, Red Cap, 100-pk.	Red	PVDF (polyvinyldifluoride)	0.2 µm	100-pk.
27898	Thomson SINGLE StEP Standard Filter Vials with Screw-Top Caps, 0.45 µm, PVDF w/Preslit Cap, Yellow Cap, 100-pk.	Yellow	PVDF (polyvinyldifluoride)	0.45 µm	100-pk.

Patent No. 7,790,117

Force C18 LC Columns

- A traditional end-capped C18 ideal for general-purpose use in reversed-phase chromatography.
- Wide pH range (2–8) provides excellent data quality for many applications, matrices, and compounds.
- High carbon load (20%) offers high hydrophobic retention.

Catalog No.	Product Name	Units
9634232	Force C18, 1.8 µm, 30 x 2.1 mm LC Column	ea.
9634252	Force C18, 1.8 µm, 50 x 2.1 mm LC Column	ea.
9634212	Force C18, 1.8 µm, 100 x 2.1 mm LC Column	ea.
963425E	Force C18, 1.8 µm, 50 x 3.0 mm LC Column	ea.
963421E	Force C18, 1.8 µm, 100 x 3.0 mm LC Column	ea.
9634332	Force C18, 3 µm, 30 x 2.1 mm LC Column	ea.
9634352	Force C18, 3 µm, 50 x 2.1 mm LC Column	ea.
9634312	Force C18, 3 µm, 100 x 2.1 mm LC Column	ea.
9634362	Force C18, 3 µm, 150 x 2.1 mm LC Column	ea.
963435E	Force C18, 3 µm, 50 x 3.0 mm LC Column	ea.
963431E	Force C18, 3 µm, 100 x 3.0 mm LC Column	ea.
963436E	Force C18, 3 µm, 150 x 3.0 mm LC Column	ea.
9634315	Force C18, 3 µm, 100 x 4.6 mm LC Column	ea.
9634365	Force C18, 3 µm, 150 x 4.6 mm LC Column	ea.
9634552	Force C18, 5 µm, 50 x 2.1 mm LC Column	ea.
9634512	Force C18, 5 µm, 100 x 2.1 mm LC Column	ea.
9634562	Force C18, 5 µm, 150 x 2.1 mm LC Column	ea.
963455E	Force C18, 5 µm, 50 x 3.0 mm LC Column	ea.
963451E	Force C18, 5 µm, 100 x 3.0 mm LC Column	ea.
963456E	Force C18, 5 µm, 150 x 3.0 mm LC Column	ea.
9634515	Force C18, 5 µm, 100 x 4.6 mm LC Column	ea.
9634565	Force C18, 5 µm, 150 x 4.6 mm LC Column	ea.
9634575	Force C18, 5 µm, 250 x 4.6 mm LC Column	ea.

Stationary Phase Category: C18, octadecylsilane (L1)
Ligand Type: End-capped C18
Particle: 1.8 µm, 3 µm, or 5 µm fully porous silica
Pore Size: 100 Å
Carbon Load: 20%
End-Cap: yes
Surface Area: 300 m^2/g

Recommended Usage:
pH Range: 2.0–8.0
Maximum Temperature: 80 °C
Maximum Pressure: 1034 bar/15,000 psi* (1.8 µm), 600 bar/8700 psi (3 µm); 400 bar/5800 psi (5 µm)

* For maximum lifetime, recommended maximum pressure for 1.8 µm particles is 830 bar/12,000 psi.

Properties:
- Compatible with moderately acidic to neutral mobile phases (pH 2–8).
- Excellent data quality in food, environmental, bioanalytical, and other applications.

Switch to a C18 when:
- You need a general-purpose column for reversed-phase chromatography.
- You need to increase retention of hydrophobic compounds.

Force Inert C18 LC Columns

- Inert LC column technology reduces nonspecific binding of chelating analytes, enabling sensitive analysis and smooth integration of peaks.
- Ideal for the analysis of metal-sensitive compounds.
- Increased response and analyte recovery, allowing lower detection limits.
- Improved peak shape without passivation or mobile phase additives.
- Part of Restek's Force Inert C18 column line featuring 1.8 and 3 µm fully porous silica.

Catalog No.	Product Name	Units
9634252-T	Force Inert C18, 1.8 µm, 50 x 2.1 mm LC Column	ea.
9634212-T	Force Inert C18, 1.8 µm, 100 x 2.1 mm LC Column	ea.
9634352-T	Force Inert C18, 3 µm, 50 x 2.1 mm LC Column	ea.
9634312-T	Force Inert C18, 3 µm, 100 x 2.1 mm LC Column	ea.
963435E-T	Force Inert C18, 3 µm, 50 x 3.0 mm LC Column	ea.
963431E-T	Force Inert C18, 3 µm, 100 x 3.0 mm LC Column	ea.

Want even better performance when analyzing metal-sensitive compounds? Check out Inert LC columns at **www.restek.com/inert**

Stationary Phase Category: C18, octadecylsilane (L1)
Ligand Type: End-capped C18
Particle: 1.8 µm or 3 µm fully porous silica
Pore Size: 100 Å
Carbon Load: 20%
End-Cap: yes
Surface Area: 300 m^2/g

Recommended Usage:
pH Range: 2.0–8.0
Maximum Temperature: 80 °C
Maximum Pressure: 1034 bar/15,000 psi* (1.8 µm); 600 bar/8700 psi (3 µm)

*For maximum lifetime, recommended maximum pressure for 1.8 µm particles is 830 bar/12,000 psi.

Properties:
- Compatible with moderately acidic to neutral mobile phases (pH 2–8).
- Excellent data quality in food, environmental, bioanalytical, and other applications.

Switch to a C18 when:
- You need a general-purpose column for reversed-phase chromatography.
- You need to increase retention of hydrophobic compounds.

Force C18 Guard Cartridge, EXP

- Free-Turn architecture lets you change cartridges by hand without breaking inlet/outlet fluid connections—no tools needed.
- Patented titanium hybrid ferrules can be installed repeatedly without compromising high-pressure seal.
- Auto-adjusting design provides ZDV (zero dead volume) connection to any 10-32 female port.
- Guard column cartridges require EXP direct connect holder (cat.# 25808).
- Pair with EXP hand-tight fitting (cat.# 25937–25938) for tool-free installation.
- For use with 3 or 5 µm Force LC columns. For 1.8 µm Force columns, use a 0.2 µm UltraShield filter.

Catalog No.	Product Name	Units
963450252	Force C18 Guard Cartridge, 5 x 2.1 mm EXP, 3-pk.	3-pk.
963450253	Force C18 Guard Cartridge, 5 x 3.0 mm EXP, 3-pk.	3-pk.
963450250	Force C18 Guard Cartridge, 5 x 4.6 mm EXP, 3-pk.	3-pk.

Force Inert C18 Guard Cartridge, EXP

- Premium inert coating reduces nonspecific binding of chelating analytes.
- Free-Turn architecture lets you change cartridges by hand without breaking inlet/outlet fluid connections—no tools needed.
- Patented titanium hybrid ferrules can be installed repeatedly without compromising high-pressure seal.
- Auto-adjusting design provides ZDV (zero dead volume) connection to any 10-32 female port.
- Guard column cartridges require EXP direct connect holder (cat.# 25808).
- For use with 3 or 5 µm Force LC columns. For 1.8 µm Force columns, use a 0.2 µm UltraShield filter.

Catalog No.	Product Name	Units
963450252-T	Force C18 Guard Cartridge, 5 x 2.1 mm EXP, 3-pk.	3-pk.
963450253-T	Force C18 Guard Cartridge, 5 x 3.0 mm EXP, 3-pk.	3-pk.